ZOOLOGICAL SCIENCE **9**: 515–527 (1992) © 1992 Zoological Society of Japan

Self-generated Zigzag Turning of *Bombyx mori* Males during Pheromone-mediated Upwind Walking

Ryohei Kanzaki[1], Naoko Sugi and Tatsuaki Shibuya

Institute of Biological Sciences, University of Tsukuba, 1-1-1 Tennodai,
Tsukuba-shi, Ibaraki 305, Japan

ABSTRACT—In order to further understand the pheromone-oriented walking of the male silkworm moth *Bombyx mori,* we have analyzed the movement of free walking males in a laboratory wind tunnel using a continuous pulsed stimulation of various frequencies with pheromones.

Single and brief pulsed stimulation (100 msec) with pheromones to either antenna elicited a long-lasting zigzag turing pattern ($\leq$4 sec) which consisted of left and right turns or vice versa. The direction of the first turn of the zigzag turning was strongly related to which antenna was stimulated. While continuous pulsed stimulation was applied, similar zigzag turnings to those triggered by a single pulsed stimulation were repeatedly observed with each stimulation. The tempo and the angle of the zigzag turning gradually increased turn by turn and were successively reduced with each stimulation.

We exposed two control mechanisms during upwind walking to the pheromone odor source: (1) a self-generated zigzag turning program triggered by a pulsed pheromonal stimulation, and (2) reset mechanisms of this program. We propose that walking *B. mori* males use similar control mechanisms for movements toward a pheromone source to those of flying moths.

INTRODUCTION

Male silkworm moths *Bombyx mori* respond to the sex-attractant pheromone released by conspecific females with a characteristic behavioral repertoire called the "mating dance" including wing vibrations, walking, and abdominal curvature [1–3]. Neurons in the brain of the male moths process information about the pheromone, integrating this information with sensory information of other modalities, then initiate and possibly regulate the motor outflow that results in oriented behavior [4–7]. Recent findings using several species of moths have indicated that, in addition to optomotor anemotaxis (steering with respect to the wind), flying males employ a self-steered program of counterturns in the process of locating the pheromone source [8–13].

It has been observed that *B. mori* [14] and *Periplaneta americana* [15] males, which exclusively or primarily walk to sources of their pheromone [14–17], execute frequent and spontaneous turns while walking in an airstream uniformly permeated with pheromones. In contrast, Willis and Baker [18] reported that *Grapholita molesta* males do not use a counterturning program while walking upwind to pheromones, whereas they do while flying upwind.

We now know that the odor plume formed downwind from the female has a highly variable structure [19, 20], and that an intermittent pheromonal stimulus greatly improves a male moth's ability to orient upwind to a pheromone source [8, 21–23]. Kramer [23] analyzed the *B. mori* walking behavior on a "Kramer's sphere" using pulsed pheromonal stimulation, and showed general features of the behavior. However, the detailed walking pattern of individual *B. mori* males has not yet been analyzed well. In order to further understand the walking pattern, in this study we analyzed the movement of free walking *B. mori* males in a laboratory wind tunnel using a continuous pulsed stimulation of various frequencies with pheromones.

We exposed two control mechanisms during upwind walking to the pheromone odor source: (1)

Accepted February 26, 1992
Received December 2, 1991

[1] To whom offprint request should be addressed

a self-generated zigzag turning program triggered by a pulsed pheromonal stimulation, and (2) reset mechanisms of this program. We propose that walking *B. mori* males use similar control mechanisms for movements towards a pheromone source to those of flying moths expected from Baker's model [22], rather than just walking directly upwind to the source.

MATERIALS AND METHODS

Insects

Bombyx mori (Lepidoptera, Bombycidae) were reared in the laboratory on an artificial diet at 25°C and 50–60% relative humidity under a 12 : 12 LD cycle, and were used within 3 days after eclosion.

Pheromonal stimulation

Two pheromonal stimuli were used in this experiment: (1) Crude pheromone extract was prepared by washing the tip of a virgin-female moth's abdomen, which contains the pheromone gland, in 100 μl of *n*-hexane (Wako reagent-grade) for 15 min, yielding "1 female equivalent (FE)" of "female extract", and (2) synthetic (*E, Z*)-10, 12-hexadecadien-1-ol, bombykol, the principal pheromone component of *Bombyx mori*, kindly provided by Shin-Etsu Chemical Co. Ltd.

Each pheromonal stimulant was applied to a piece of filter paper (1×2 cm), which was then inserted into a plastic cartridge (1 ml). The cartridge was placed in the wind tunnel 1 cm above the floor and upwind. Pulsed olfactory stimulation was produced by switching the clean air stream to the stimulant cartridge, which was controlled by a 3-way solenoid valve [24]. The frequency (0.25–2 Hz) and duration (100 msec) of the stimulation was controlled by an electronic stimulator. Odorants were thus introduced into the wind tunnel (1.67 ml/100 msec). The stimulation was monitored by later video observation of a synchronized green light-emitting diode (LED) connected to the electronic stimulator, which was on the floor of the wind tunnel.

Wind tunnel and data recording

The wind tunnel was constructed using clear polycarbonate plastic which had a working section 49 cm long, 49 cm wide at floor level, and 32 cm high. Air flow was introduced into the tunnel by negative pressure generated by a voltage regulated fan. The odors used were removed from the room by an exhaust duct attached to the fan at the downwind end of the tunnel. The wind velocity was 0.5 m/sec, which was determined by measuring the flow rate of the exhausted air with a flow meter. The shape and position of the pheromone plume was simulated by applying $TiCl_4$, which makes a smoke plume, to the same size filter paper as used for pheromone sources, and then placing it in the same location in the wind tunnel as the pheromone would be during an experiment.

To prevent pheromonal contamination on the floor of the wind tunnel, the brown paper on the floor was replaced for every experiment. Individual males were placed on the center of the plume 20–25 cm away from the tip of the stimulant cartridge. A continuous clean air stream did not arouse males on the floor of the wind tunnel. The room temperature was approx. 26°C.

Walking tracks of individual males were recorded on a Sony SLO-333 video recorder using a Sony HVC-80 video camera positioned above the wind tunnel. All recordings were played back frame-by-frame through a 53 cm (21 inch) NEC video monitor. The computer (NEC PC-9801VX) screen was superimposed on the video monitor by a video-converter system and the consecutive locations of the head and tail of each moth were digitized and input every 0.1 sec into the computer by pointing the position by moving a 'mouse' cursor on the screen and pushing the 'mouse' button. The position, walking speed, and body axis with respect to the upwind direction of each moth were calculated by the computer.

RESULTS

Upwind walking to various frequencies of continuous pulsed pheromonal stimulation

Continuous pulsed pheromonal stimulation was applied to free walking *Bombyx mori* males to determine the affect of the frequency of the stimulation on the upwind walking movement. In these experiments the duration of the stimulation was constant (100 msec). Female extract (1 FE) was used as pheromonal stimulant. In some cases 0.1–10 μg synthetic bombykol was used. Approximately 1 μg of bombykol was found to be present in 1 FE of solution using gas chromatographic analysis (data not shown). Both stimulants affected the initiating walking (197 out of 200 trials (98.5%) in 35 males, Table 1), of which >85% (in average) located the source (Table 1). All males

TABLE 1. Percent of *Bombyx mori* males responding to different frequencies of continuous pulsed stimulation with pheromone

frequency^{*1} (Hz)	n	%initiating walking	of those initiating walking, %locating upwind	of those locating upwind, %showing the pattern	turns per^{*2} stimulation
constant^{*3}	17	100.0	88.2	0.0	nt
2.00	66	97.0	93.8	0.0	0.12
1.00	40	97.5	94.9	33.3	0.71
0.50	34	100.0	91.2	94.1	1.28
0.33	22	100.0	86.4	77.3	nt
0.25	21	100.0	76.2	90.5	2.36

^{*1} Duration of the stimulation was constant (100 msec).

^{*2} Total number of turns were divided by the number of stimulation.

^{*3} Constnant stimulation was introduced into the air stream.

nt: not tested.

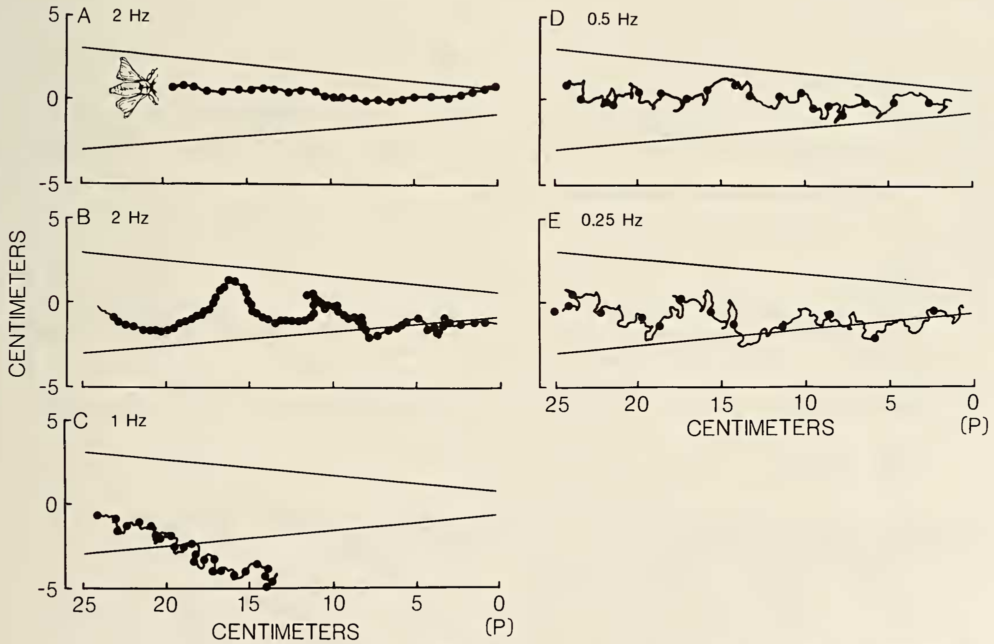

FIG. 1. Plots of the tracks, as viewed from above, *B. mori* males walking upwind to various frequencies (Hz) of continuous pulsed pheromonal stimulation (1 FE). Duration of the stimulation was 100 msec. Wind was from the right (0.5 m/sec). Pheromonal stimulation was introduced into the air stream of the wind tunnel as (A) 2 Hz, (B) 2 Hz, (C) 1 Hz, (D) 0.5 Hz, (E) 0.25 Hz pulses. Filled circles indicate the head position when the olfactory stimulation was introduced into the air stream. Plots of B-E are of the same moth. Lines indicate the average boundaries of the pheromone plumes simulated by $TiCl_4$ smoke. Outer borders are the dimensions of the wind tunnel and the diagrams are of linear scale from the pheromone source (P).

walking upwind did so while fluttering their wings. No significant differences in the walking movement were observed while using female extract or synthetic bombykol. In a few cases, males did not walk all the way to the source but stopped and sat in the plume area or turned away from the plume and sat outside it (e.g., Fig. 1C).

Figure 1 shows plots of tracks of a male moth walking upwind in response to various freqeuncies of continuous pulsed stimuli with pheromones (1 FE). A clean air pulse introduced into the wind tunnel did not arouse males (data not shown). When male moths received a constant or a high frequency (e.g., 2 Hz) of pheromonal stimulation, most walked in nearly a straight line, steering directly upwind (Fig. 1A). In a few cases they walked at angles between 20° and 50°, and sometimes more than 70° oblique to the direction of upwind (Fig. 1B), as described by Kramer [14]. Although their side to side movement was less than their wing-span (approximately 4 cm, Fig. 1A), the angle of the body axis with respect to upwind (0°) changed dramatically as the frequency of pulsed stimulation was reduced (Figs. 1, 2).

A turn was defined as when the body axis changes from clockwise to anticloskwise (or vice versa) of more than 10° and the time interval (tempo) of the turn was more than 0.2 sec, in order to eliminate angular error during digitizing.

Figure 2 shows the angles of the body axis with respect to upwind plotted against times. In 0.25 Hz stimulation (Fig. 2E), when the male received the stimulation puff while the angle of the body axis was between 0° and 90°, the male attempted to turn to the right and flipped to the left. On the other hand, males showed symmetrical movements when the angle was between 270° and 360°; i.e., they turned to the left then flipped to the right. The tempo of the first turn elicited just after the stimulation was 1.1±0.1 sec (mean±SE, n=48, Table 3). The angle of the turn was 68±5° (n=48, Table 3). Each time a male received the stimu-

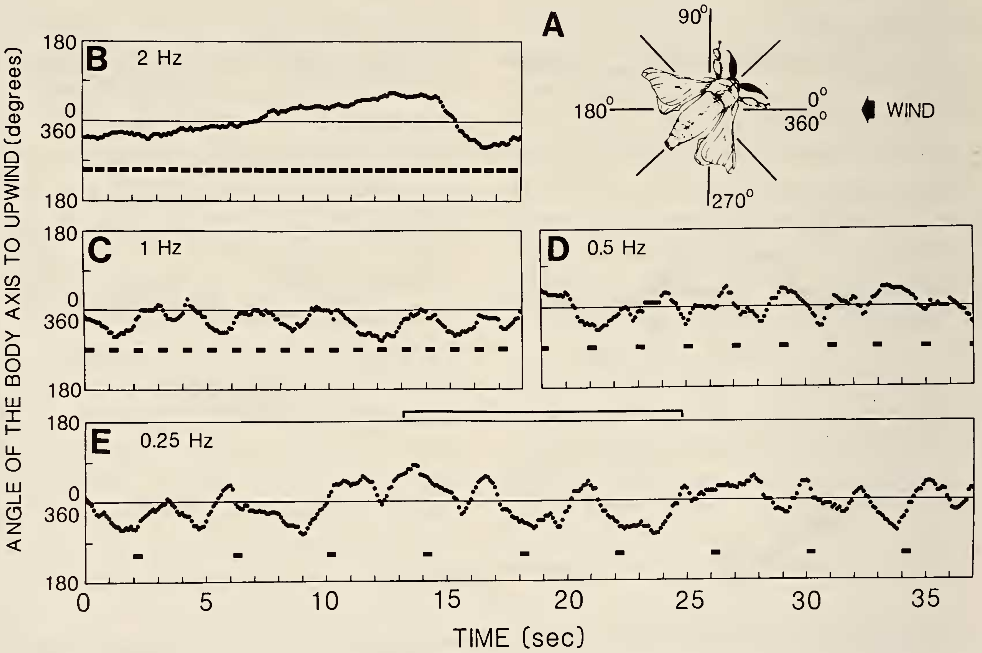

Fig. 2. The angles of the body axis with respect to upwind (0°) plotted against time when the male was in the pheromone plume. Replotted from the same data shown in Fig. 1. (A) The angle of the body axis of the male is measured with respect to upwind. The angle of the male in (A) is 45°. The arrow indicates the wind direction. (B-E) Plots of the angle against time when a various frequency of continuous pulsed stimulation was applied. Solid lines beneath plots represent the period of olfactory stimulation. The region of plot (E) indicated by the bar is expanded in Fig. 4B.

lation at any phase of the turn, the switching of the movement occurred. More than 85% (in average) of males which were locating upwind showed this zigzag turning pattern when the frequency was between 0.5 and 0.25 Hz (Table 1). Thus, the direction of the first turn elicited by the stimulation was strongly related to the angle of the body axis to upwind. The zigzag turning pattern was repeatedly observed when the frequency was reduced (Fig. 2C-E).

We then, applied continuous pulsed stimulation to males for which an antenna had been removed completely from the basal segment; such males could receive the pheromones only by one intact antenna. In 0.25 Hz stimulation, most of the treated males could not orient to the pheromone source. Although they showed a similar zigzag turning pattern when the antenna had been stimulated, they turned away from the plume. It may be because that the treated males usually began to turn in the same direction as the intact antenna and the possibility to turn away from the plume became high. In some cases, as shown in Fig. 3B, the male succeeded to orient to the source with showing a typical zigzag pattern similar to the intact males. By contrast, when a treated moth received 2 Hz stimulation, >80% of treated males could orient to the source as shown in Fig. 3A. In this case, at first the male continued to turn in the same direction as the intact antenna, and then walked in nearly a straight line, steering directly upwind. thus, such treated males showed a similar zigzag turning pattern to intact ones.

The average number of turns per stimulation is shown in Table 1. Males showed 2.36 turns on the average per stimulation of 0.25 Hz. The number of turns decreased with increases in frequency. With a stimulation frequency of 2 Hz, turning was rarely observed (0.12/stimulation). Thus, the zigzag turning pattern gradually disappeared with increased frequency of pulsed olfactory stimulation.

Figure 4 illustrates the walking tracks and the angles of the body axis with respect to upwind against time: Plots of the same data are shown in Fig. 2E (solid line above the plots). In some cases, before showing the turning pattern, males walked virtually straight along the body axis (i.e., their body axis angle changed only slightly compared with the zigzag turns). This straight line walking was also elicited in response to higher frequencies of stimulation (0.5–1 Hz) and lasted ≤0.5 sec, and then the zigzag turns followed this straight line walking (Figs. 2–4). In many other cases, how-

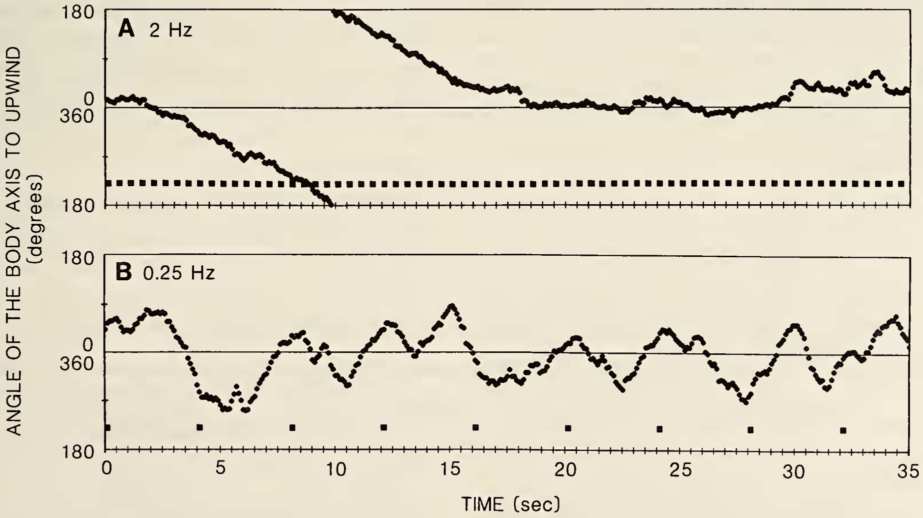

Fig. 3. The angles of the body axis of the treated males for which the left antenna was completely removed from the basal segment, are plotted against time. (A) 2 Hz pulsed pheromonal stimulation. (B) 0.25 Hz pulsed stimulation. Plots of (A) and (B) are of the different moth. Even treated males showed zigzag turnings in response to the stimulation. Solid lines beneath plots represent the period of stimulation (1 FE).

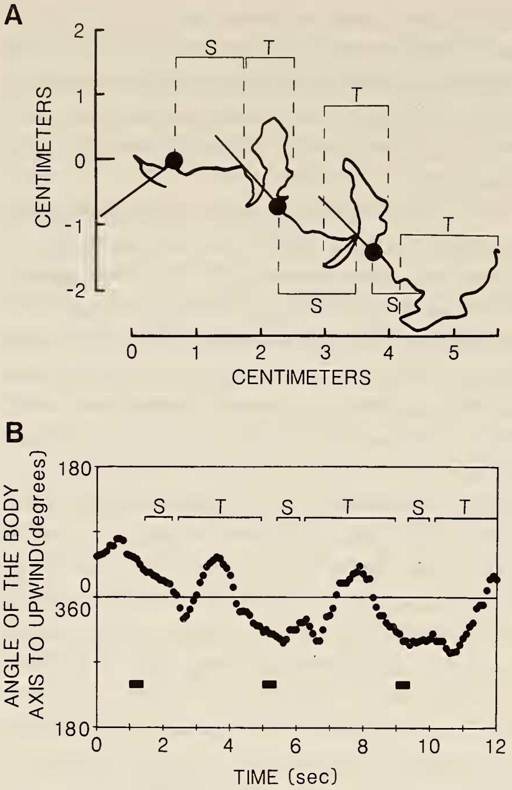

FIG. 4. Plots of the tracks (A) and the angles of the body axis with respect to upwind, are plotted against time (B): Same data as in Figs. 1E and 2E. In some cases, *B. mori* males walked virtually straight along the body axis (S) before typical zigzag turnings (T). This straight line walking lasted ≤0.5 sec. Filled circles in (A) indicate the head position when the olfactory stimulation was introduced into the air stream. Solid lines from the filled circles indicate the body axis. Solid lines beneath plots in (B) represent the stimulation.

ever, it was difficult to distinguish any brief straight line walking from the turns. It is sometimes observed that the walking speed became relatively higher when the moths showed the straight line walking (data not shown).

Males whose eyes were pained black to prevent visual affects on the movement could also orientate towards the goal with a similar zigzag walking pattern (data not shown) [25].

Single pulsed pheromonal stimulation to either antenna

In order to know the detail of this zigzag turning pattern against time, single pulsed pheromonal stimulation (100 msec of duration) was applied to either antenna of a resting male on the open field without wind (Fig. 5). The stimulant cartridge was placed in front of either antenna approximately 1 cm apart. In 170 out of 172 trials (98.8%) using 29 males, single pulsed stimulation elicited initiation of walking (Table 2). 87.3% (in average) of those initiating walking showed identical zigzag turning patterns to those triggered by a low frequency of pulsed stimulation; that is, the male showed back and forth turning by applying the pulsed stimulation to the antenna (e.g., Fig. 5Aa, b). We predict that with single pulsed stimulation there is a temporal difference in the pheromone concentration and/or a delay in signal reception between one antenna and the other. To prevent contamination of the stimulant to the other antenna, we also used treated males for which one antenna had been removed from the basal segment as described previously (Figs. 3, 5Ac, d).

Figure 5B illustrates relative changes of the angle of the body axis against time with single

TABLE 2. Percent of *Bombyx mori* males responding to single pulsed stimulation with pheromone

stimulation[*1]		n	%initiating walking	of those initiating walking %showing the pattern
intact	left	71	98.6	84.3
	right	68	98.5	77.6
treated	left	13	100.0	95.0
	right	20	100.0	92.3

[*1] Treated males for which other antenna was removed completely from the basal segment.

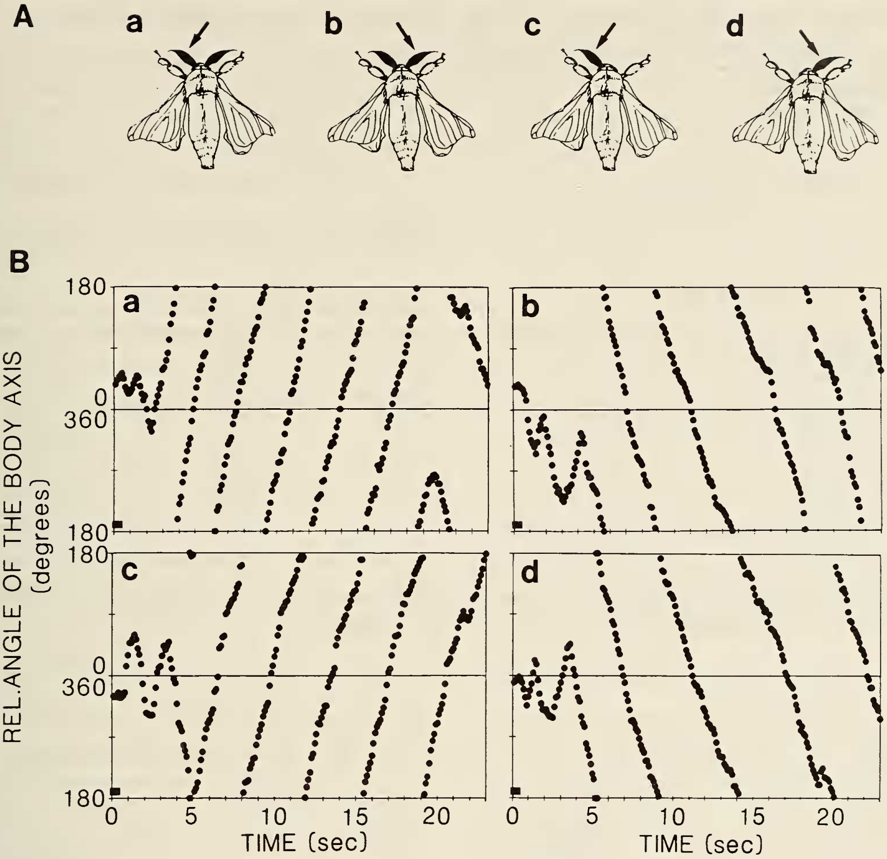

FIG. 5. Single pulsed stimulation (100 msec) with pheromones (1 FE) was applied to the antenna. (A) Stimulation was applied independently to the left antenna (a, c) and right antenna (b, d) of the intact moths (a, b) and treated moths (c, d) for which one antenna had been removed completely from the basal segment. Arrows indicate the direction of the stimulation. (B) Plots of the relative angles of the body axis against time when single pulsed stimulation was applied to the antenna as shown in (A). Plots of (a, b) and (d) are of the same moth. Solid lines beneath the plots in (B) indicate the stimulation period.

pulsed stimulation (1 FE). For approximately 4 sec after the stimulation, both treated males (Fig. 5Ac, d) and intact males (Fig. 5Aa, b) showed a turning pattern which was identical to the pattern shown in Fig. 4B. A symmetrical zigzag turning pattern was elicited by applying the pheromones to one antenna or the other (Fig. 5B). When the stimulation was applied to the left antenna (Fig. 5Aa, c), the male turned to the left first, then flipped to the right (Fig. 5Ba, c), while the male turned to the right first, then flipped to the left, when the stimulation was applied to the right antenna (Fig. 5Ab, d, 5Bb, d). More than 80% (in average) of intact males and >90% (in average) of treated males showed similar turning patterns (Table 2). The tempo of the first turn was 1.2±0.1 sec (mean±SE, n=16, Table 3). The angle of the first turn was 91±14° (n=16, Table 3). Other males stopped walking before complete turning and a few males turned in the opposite direction from the stimulated antenna.

In general, the direction of the first turn depends strongly on the antenna stimulated with pheromones. A brief straight line walking was sometimes observed in this set of experiments. After males showed the zigzag turning pattern two or three times, the pattern was shifted to a "looping" (turns of more than 360° or greater). Seven out of

TABLE 3. Mean and SE of tempo and angle of the zigzag turn in response to single pulsed stimulation and 0.25 Hz pulsed stimulation

Single Pulsed Stimulation		1st turn	2nd turn	3rd turn
tempo	abs^{*1}	1.2 ± 0.1^{a}	1.9 ± 0.3^{b}	2.1 ± 0.4^{b}
(sec)	rel	1^{a}	1.5 ± 0.2^{b}	2.4 ± 0.4^{c}
angle	abs	91 ± 14^{a}	168 ± 31^{b}	181 ± 35^{b}
(degrees)	rel	1^{a}	1.8 ± 0.2^{b}	3.2 ± 0.4^{c}
n		16	16	8

0.25 Hz Pulsed Stimulation		1st turn	2nd turn	3rd turn
tempo	abs	1.1 ± 0.1^{a}	1.6 ± 0.1^{b}	1.7 ± 0.1^{b}
(sec)	rel	1^{a}	1.5 ± 0.1^{b}	1.9 ± 0.1^{c}
angle	abs	68 ± 5^{a}	121 ± 10^{b}	132 ± 12^{b}
(degrees)	rel	1^{a}	2.0 ± 0.2^{b}	2.7 ± 0.3^{b}
n		48	48	29

Means in the same row having no letters in row are significantly different according to t-test ($P < 0.05$).
$*^{1}$ abs: absolute time or angle; rel: relative to the 1st turn

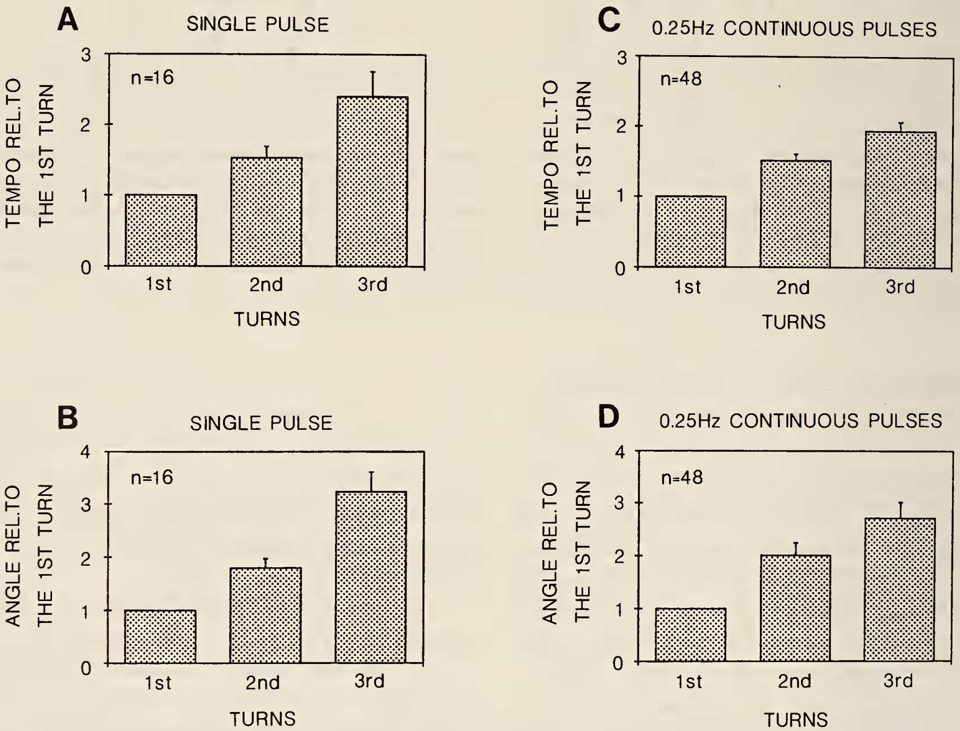

FIG. 6. Histograms of the relative tempo (A, C) and angles (B, D) of each turn of the zigzag turns, which were elicited in response to single pulsed stimulation (A, B) and 0.25 Hz pulsed stimulation (C, D). All the values were calculated by the computer and the mean and standard error are indicated.

10 (70%) males tested looped in the same direction as the antenna stimulated. One way looping continued for more than 10 sec (Fig. 5B). Then, the direction of the looping was irregularly changed (e.g., Fig. 5Ba). In many cases the total period of looping lasted more than 30 sec.

Changes of tempo and angle of the zigzag pattern against time

Changes of tempo and angle of the zigzag turning pattern elicited by 0.25 Hz (e.g., Fig. 2E) and single pulsed stimulation (e.g., Fig. 5B) with pheromones were measured against time (Table 3). These two parameters (i.e., tempo and angle) of each turn were compared with those of the first turn which occurred after each stimulation. The results are represented as histograms in Fig. 6.

Figures 6A and 6B illustrate the relative changes of the tempos and angles turn by turn in response to a single pulsed stimulation to either antenna. The average tempos relative to the first turn were 1.5±0.2 (mean±SE, n=16) in the second turn and 2.4±0.4 (n=8) in the third turn. The average angles were 1.8±0.2 (n=16) in the second turn and 3.2±0.4 (n=8) in the third turn. The values of both tempo and angle were significantly different turn by turn (*t*-test, $P<0.05$). Thus, the values of both parameters in each turn were increased turn by turn (Table 3).

Figures 6C and 6D illustrate the relative changes of the tempos and angles turn by turn in response to 0.25 Hz pulsed stimulation. The average tempos relative to the first turn were 1.5±0.1 (n=48) in the second turn and 1.9±0.1 (n=29) in the third turn. The average angles were 2.0±0.2 (n=48) in the second turn and 2.7±0.3 (n=29) in the third turn. The values of the tempo were singificantly different turn by turn ($P<0.05$). The values of the angles between the first turn and the second turn were significantly different ($P<0.05$). Thus, the values of each parameter were increased turn by turn between the stimuli as in the case of single pulsed stimulation. However, each value was successively reduced by the new pulsed stimulation. Then, each value again, gradually increased turn by turn until the next stimulation (Fig. 6C, D). Similar characteristics were observed on the treated males for which one antenna was completely removed from the basal segment (Fig. 3B). These results imply that the zigzag turning pattern, which was triggered by a brief pheromonal stimulation, is "reset" every time the new pheromonal stimulation is applied to either antenna. In addition, the reset mechanisms occurred at any phase of the turning (Figs. 2, 3).

DISCUSSION

Kramer [23] previously investigated similar behavioral experiments as this study using his "Kramer's sphere". In his study, he averaged a great number of runs on the sphere and showed the general features of the pheromone-oriented walking of *B. mori* males. In this study, we have analyzed a detailed walking pattern of individual male walking freely in a laboratory wind tunnel using a continuous pulsed stimulation of various frequencies with pheromones.

Self-generated zigzag turning program

Single pulsed stimulation (100 msec) with pheromones to either antenna exposed a zigzag turning pattern which consisted of left and right turns or vice versa, as shown in Fig. 5. Even the treated males for which one antenna was removed showed a similar zigzag turning pattern in response to the single pulsed stimulation (Fig. 5Bc, d). Brief pulsed stimulation elicited the zigzag turning pattern repeatedly for a longer period (approximately 4 sec) than the stimulation period (100 msec): We did not have to keep applying the constant stimulation to the male during the zigzag turning pattern. The tempo and angle of the zigzag turning gradually increased turn by turn when the male was stimulated with pulsed pheromones (Figs. 5, 6A, B, Table 3).

It has been reported in flying moths that the tempo at which turns occur becomes progressively longer during "casting" flight and that turns persist for seconds when the moth loses contact with the pheromone plume [11, 13]. We propose that walking moths also have a self-generated zigzag turning program with characteristics similar to the counterturning program of the "casting" flight.

In this study, we have not examined the affect of the wind speed and pheromone concentrations on the zigzag walking pattern. In order to further understand the program, we must clarify the affect of these factors on the program [23].

Although Kramer [23] showed a rate of change of direction during the walk and the cosine of the walking direction with respect to the upwind, such a zigzag turning found in this study, was not detected by his sphere experiments. This difference might have any of several explanations: (1) We have analyzed an movement of an individual moth, whereas in the Kramer's experiments, a great number of runs were averaged. (2) Kramer averaged a rate of change of direction and the cosine of the walking direction, whereas, we have analyzed the actual changes of the body axis of an individual moth (i.e., the tempo and the angle). (3) Discrepancies might be due to the differences in the experimental methods, i.e., we have used a freely walking moth in the wind tunnel, whereas a moth was on the Kramer's sphere.

As illustrated in Fig. 5 the direction of the first turn of this zigzag turning was strongly related to which antenna was stimulated with pheromones. It is possible that the direction of the first turn may be related to the concentration differences and/or delay between one antenna and the other. However, Kramer [23] suggested it was unlikely that the *B. mori* males detected and utilized small differences between the time of stimulation of the two antennae. He did not report the direction of the first turn just after the stimulation. Again, these differences may reflect differences in experimental methods and so on described above.

It is reported by Kramer [23] that the pulsed stimulation increased the walking speed of *B. mori* rapidly. We have measured the changes of the walking speed of an individual *B. mori*. It is sometimes observed that the walking speed became relatively higher when the moths showed the straight line walking just after the stimulation.

"Reset mechanisms" of the zigzag turning program

As illustrated in Fig. 6C, D. and Table 3, when continuous pulsed stimulation (0.25 Hz) was applied during upwind walking, the tempo and the angle of the turn just before the stimulation were successively reduced with each stimulation, and then the tempo and angle gradually increased turn by turn until the next stimulation. Similar characteristics were observed on the treated males for which one antenna was completely removed from the basal segment (Fig. 3B). These results imply that the zigzag turning program, which was triggered by a brief pheromonal stimulation, is "reset" every time the new pheromonal stimulation is applied to either antenna. In addition, the reset mechanisms occurred at any phase of the turning (Figs. 2, 3).

Straight line walking during higher frequencies of pulsed stimuli with pheromones

We found that some males walked in almost straight lines along the body axis for ≤ 0.5 sec just after each of the continuous pulsed stimuli, and the zigzag turning pattern followed to the brief straight line walking (Fig. 4). The results of continuous pulsed stimulation indicated that the turning pattern gradually disappeared with increases in the stimulation frequency and that males walked in almost straight paths, steering directly upwind (Figs. 1A, 2B, 3A, Table 1). Kramer [23] reported similar results in a male *B. mori* with the "Kramer's sphere" experiments.

We predict that as the frequency of the stimulation is increased, the "reset mechanisms" as described above and a "brief straight line walking" occur more frequectly. Under this condition, changes in the angle of the body axis may become smaller, until finally the axis is almost directly upwind. This might be part of the reason why the males may walk in almost a straight line when the frequency is higher (i.e., 2 Hz).

Willis and Baker [18] reported that male *Grapholita molesta* do not use a counterturning program while walking upwind to pheromone, whereas they do while flying upwind. Constant olfactory stimulation, or in other words, high frequency of pulsed stimulation, was applied in their experiments. We are very interested in whether *G. molesta* males show similar zigzag walking patterns to those observed in *B. mori* males when continuous low frequency pulsed stimulation is applied.

On the other hand, when the frequency of the pulsed stimulation is lower (e.g., <0.5 Hz), we propose that following things happen on the male as a part of the pheromone-oriented mechanisms of *B. mori* males. The pulsed pheromonal stimulation triggers the zigzag turning program including the straight line walking. Then, the male begins to display a zigzag turning pattern. The first turn of the zigzag turns is strongly related to which antenna is stimulated. The tempo and the angle of the turn are gradually increased turn by turn until the next stimulation is applied to the antenna. When the next new pulse stimulates the antenna, the zigzag turning program is "reset" and the male begins to turn to the same direction as the antenna stimulated, and then repeats the procedure.

The upwind walking of *B. mori* males mediated by pheromonal stimulation may contain several control mechanisms. In this study we found that the upwind walking contains at least following two control mechanisms: (1) a self-generated zigzag turning program, and (2) reset mechanisms of this program. And the direction of the first turn of this zigzag turning might be strongly related to which antenna is stimulated with pheromones.

As described above, it is possible that the direction of the first turn may be related to the concentration differences and/or delay between one antenna and the other. While, treated males, for which one antenna had been removed, also showed similar characteristics of zigzag turnings to intact ones (Figs. 3, 5). For example, some of the treated males walked in nearly a straight line, steering directly upwind in 2 Hz stimulation (Fig. 3A) and in 0.25 Hz stimulation, they showed a typical zigzag turning and oriented toward the odor source (Fig. 3B). Though the treated males could not detect the concentration differences or delay between one antenna and the other, they could detect the upwind and orient toward the pheromone source. This suggests that the male moths may have some other upwind detecting mechanisms while orienting toward the odor source.

Walking moths vs. flying moths

Baker [11] proposed a model to explain the generation of zigzagging upwind flight in moths. This model invokes the complementary action of a dual flight control system in which contact with pheromone filaments in the turbulent plume would produce: (1) longer-lasting activation of a counterturning program, which would continue to produce the turns that constitute "casting" flight, and (2) rapidly activated surges of upwind flight that would also rapidly cease upon the next encounter with clean air.

These two control mechanisms seem similar to those we found in *B. mori* males in this study; i.e., (1) a self-generated zigzag turning program and (2) "reset" mechanisms of the program and a brief straight line walking. We still do not know whether this brief straight line walking occurs every time stimulation is applied. However, results of this study basically support Baker's model; that is, it is possible that a similar control system exists in both walking *B. mori* males and flying moths while they are orienting to the pheromone source.

The zigzag turning pattern was sustained for approximately 4 sec after the pheromonal stimulation was applied to either antenna (Fig. 5B). This turning was elicited even by a single and brief (100 msec) pulsed stimulation. Therefore, it is suggested that some neurons in the central nervous system maintain this neural information. Long-lasting increases in firing elicited by pheromones have been described by Olberg [4] in a class of descending neurons (DNs), which project from the brain toward the thoracic motor center, termed "flip-flopping" interneurons, in male *B. mori*. These neurons exhibited conditional or state dependent responses, in which stimuli applied when a neuron was in a state of low-frequency firing elicited accelerated firing, while identical stimuli applied when the neuron was in a state of high-frequency firing caused decelerated firing (hence the term flip-flop). The activity states correlated with changes in turnings during the olfactory-mediated walking. Olberg [4] did not report the initial activity changes of the flip-flop activity. We are interested in the activity changes of these DNs when a short pulsed olfactory stimulation is applied to either antenna. While, in flying male moths *Manduca sexta*, Kanzaki *et al.* [24] have reported DNs which show state-dependent activities to stimulation of the antenna by their pheromone blend. Another group of DNs has been physiologically and morphologically identified by

Kanzaki *et al.* in *B. mori* [7] and *M. sexta* [24] that showed long-lasting excitation (LLE) responses to stimulation of the antenna by pheromones, but did not give conditional responses similar to flip-flopping. The morphology of these DNs are similar in both species [7, 24]. These LLE responses were elicited only by pheromonal stimulation in both *B. mori* [7] and *M. sexta* [24]. Stimulation of other modalities (e.g. visual and mechanosensory stimuli to the antennae) did not elicit such responses [7, 24]. Thus, similar neural activities exist in pre-motor DNs of both walking and flying moths. This also supports that the orientation of walking and flying moths may be controlled by similar neural systems.

B. mori males usually walk with fluttering their wings and they really try to fly, but their bodies are obviously too heavy. We know that they use the wing system similar to flying moths *Manduca sexta* [26–28] after the analysis of the wing movements (especially wing remote) using a high-speed video camera system (1000 frames/sec), and they show similar zigzag turning only by their wings when their legs have been completely removed [Kanzaki *et al.* unpublished observation]. It seems that the degeneration of the flight system of *B. mori* males may not have gone. We predict, therefore, that the zigzag turning program also affects the wing system of the *B. mori.*

Using intracellular recording and staining methods, we hope to clarify the role(s) of LLE and state-dependent activity changes of olfactory DNs in the generation of motor activity (i.e., the zigzag turning program) underlying pheromone-mediated upwind walking with fluttering.

ACKNOWLEDGMENTS

This research was partially supported by Grant-in-Aid from the Ministry of Education, Science and Culture (Nos. 02640547, 03304010), Grant-in-Aid from Bio-Media Program of the Ministry of Agriculture, Forestry and Fisheries of Japan (BMP 91-I-2-4) and also for Insect Pheromone Research from the Shin-Etsu Chemical Co. Ltd.

REFERENCES

1 Schneider, D. (1969) Insect olfaction: deciphering system for chemical messages. Science, **163**: 1031–1037.

2 Kaissling, K. E. (1971) Insect olfaction. In “Handbook of Sensory Physiology IV, Chemical Senses, Olfaction”. Ed. by L. M. Beidler, Springer-Verlag, Berlin, pp. 351–431.

3 Obara, Y. (1979) *Bombyx mori* mating dance: an essential in locating the female. Appl. Ent. Zool., **14**(1): 130–132.

4 Olberg, R. M. (1983) Pheromone-triggered flip-flopping interneurons in the ventral nerve cord of the silkworm moth, *Bombyx mori.* J. Comp. Physiol., **152**: 297–307.

5 Olberg, R. M. (1983) Interneurons sensitive to female pheromone in the deutocerebrum of the male silkworm moth, *Bombyx mori.* Physiol. Entomol., **8**: 419–428.

6 Kanzaki, R. and Shibuya, T. (1986) Identification of the deutocerebral neurons responding to the sexual pheromone in the male silkworm moth brain. Zool. Sci., **3**(3): 409–418.

7 Kanzaki, R. and Shibuya, T. (1986) Descending protocerebral neurons related to the mating dance of the male silkworm moth. Brain Res., **377**: 378–382.

8 Kennedy, J. S., Ludlow, A. R. and Sanders, C. J. (1981) Guidance of flying male moths by wind-borne sex pheromone. Physiol. Entomol., **6**: 395–412.

9 Kuenen, L. P. S. and Baker, T. C. (1983) A non-anemotactic mechanism used in pheromone source location by flying moths. Physiol. Entomol., **8**: 277–289.

10 Baker, T. C., Willis, M. A. and Phelan, P. L. (1984) Optomotor anemotaxis polarizes self-steered zigzagging in flying moths. Physiol. Entomol., **9**: 365–376.

11 Baker, T. C. (1990) Upwind flight and casting flight: Complementary phasic and tonic system used for location of sex pheromone sources by male moths. In “Proc. 10th Int. Symp. Olfaction and Taste”. Ed. by K. B. Døving, Oslo, pp. 18–25.

12 Kanzaki, R. and Arbas, E. A. (1990) Olfactory and visual influences on fictive flight in *Manduca sexta.* Soc. Neurosci. Abstr., **16**: 758.

13 Willis, M. A. and Arbas, E. A. (1991) Odor-modulated upwind flight of the sphinx moth, *Manduca sexta* L. J. Comp. Physiol. A **169**: 427–440.

14 Kramer, E. (1975) Orientation of the male silkmoth to sex attractant bombykol. In “Olfaction and Taste V”. Ed. by D. A. Denton and J. P. Coghlan, Academic Press, New York, pp. 329–335.

15 Tobin, T. R. (1981) Pheromone orientation: role of internal control mechanisms. Science, **214**: 1147–1149.

16 Tobin, T. R. and Bell, W. J. (1982) Guidance system for pheromone orientation in moths. Nature, **295**: 263.

17 Preiss, R. and Kramer, E. (1986) Anemotactic orientation of gypsy moth males and its modification by the attractant pheromone (+)-disparlure during walking. Physiol. Entomol., **11**: 185–198.

18 Willis, M. A. and Baker, T. C. (1987) Comparison of manoeuvres used by walking versus flying *Grapholita molesta* males during pheromone-mediated upwind movement. J. Insect Physiol., **33**(11): 875–883.

19 Murlis, J. (1986) The structure of odour plumes. In "Mechanisms in Insect Olfaction". Ed. by T. L. Payne, M. C. Birch and C. E. J. Kennedy, Oxford University Press, Oxford, pp. 27–38.

20 Murlis, J. Willis, M. A. and Cardé, R. T. (1990) Odour signals: patterns in time and space. In "Proc. 10th Int. Symp. Olfaction and Taste". Ed. by K. B. Døving, Oslo, pp. 6–17.

21 Willis, M. A. and Baker, T. C. (1984) Effects of intermittent and continuous pheromone stimulation on the flight behaviour of the oriental fruit moth, *Grapholita molesta*. Physiol. Entomol., **9**: 341–358.

22 Baker, T. C. (1986) Pheromone-modulated movements of flying moths. In "Mechanisms in Insect Olfaction". Ed. by T. L. Payne, M. C. Birch, C. E. J. Kennedy, Oxford University Press, Oxford, pp. 39–48.

23 Kramer, E. (1986) Turbulent diffusion and pheromone-triggered anemotaxis. In "Mechanisms in Insect Olfaction". Ed. by T. L. Payne, M. C. Birch and C. E. J. Kennedy, Oxford University Press, Oxford, pp. 59–67.

24 Kanzaki, R., Arbas, E. A. and Hildebrand, J. G. (1991) Physiology and morphology of descending neurons in pheromone-processing olfactory pathways in the male moth *Manduca sexta*. J. Comp. Physiol. A, **169**: 1–14.

25 Charlton, R. E. and Cardé, R. T. (1990) Orientation of male gypsy moths, *Lymantria dispar* (L.), to pheromone sources: the role of olfactory and visual cues. J. Insect Behav., **3**(4): 443–469.

26 Kammer, A. E. (1967) Muscle activity during flight in some large Lepidoptera. J. Exp. Biol. **47**: 277–295.

27 Kammer, A. E. (1971) The motor output during turning flight in a hawkmoth, *Manduca sexta*. J. Insect Physiol. **17**: 1073–1086.

28 Rheuben, M. B. and Kammer, A. E. (1987) Structure and innervation of the third axillary muscles of *Manduca* relative to its role in turning flight. J. Exp. Biol. **131**: 373–402.